JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Model Study on the Effect of 15 Phenolic Olive Mill Wastewater Constituents on Seed Germination and *Vibrio fischeri* Metabolism

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Olive mill wastewaters (OMW) can be a severe problem when disposed of as untreated because of their high organic load, elevated concentration of polyphenols, and moderately low biodegradability. In the present study, the acute toxicity of 15 compounds with low molecular weight (<350 Da), catechol, four benzoic acids, three phenylacetic acids, three phenylethanols, and four cinnamic acids, already isolated from the reverse osmosis in the fractionation of OMW, was assessed on the marine bacterium *Vibrio fischeri* and on the seeds of two dicotyledonous species *Cucumis sativus* and *Lepidium sativum*, and on one monocotyledon *Sorghum bicolor*. Results of phytotoxicity showed that the most toxic compounds were catechol (EC_{50}s ranging from 0.40 mmol/L for *S. bicolor* to 1.09 for *C. sativus*) and hydroxytyrosol, (EC_{50}s ranging from 0.47 mmol/L for *S. bicolor* to 1.55 for *C. sativus*) while the toxic potential on bacteria was particularly elevated with EC_{50} values 1 or 2 orders less than phytotoxicity. These results suggested that the risk of OMW disposal may be more elevated for the water compartment than for the soil.

KEYWORDS: Olive mill wastewater; phenolic compounds; phytotoxicity; *Vibrio fischeri*

INTRODUCTION

Olive mill wastewater (OMW) management is a serious environmental issue for the Mediterranean area where there is the most production of olive oil. OMW is characterized by a significant toxicity for aquatic and terrestrial organisms because of its high organic load, low biodegradability (*1*), and elevated concentration of phenol components (*2*, *3*). Consequently, a direct biological treatment of OMW in urban sewage treatment plant (STP) is not feasible, and various pretreatment techniques have been successfully used to diminish the effect of this waste on STP (*2*, *4*, *5*). On the other hand, OMW is often discharged in the environment by means of its spreading on agricultural soils when applied at appropriate OMW/soil surface rates. In that case, despite a certain biodegradative capability of soil (*6*, *7*), some suggestions in OMW spreading have to be considered for its direct use such as amounts in m^3 ha^{-1} $years^{-1}$ (*7*), dilution, and aeration (*8*). In fact, the phytotoxic properties of OMW are well-known but, when high-tech OMW treatments are not possible, its spreading on the land may be considered as an applicable system to avoid the direct release in surface waters (*8*, *9*).

Examining the characteristics of OMW, there is to consider the serious complexity because of its seasonal discharge (from November to February) and the large volume of wastes limited at the Mediterranean area (Greece, Italy, Spain, Tunisia) with an esteemed effluent production of about 3×10^7 m^3 (*9*−*11*) equivalent to the pollution produced by more than 22 million people $years^{-1}$ (*12*). At the moment, for every liter of oil, produced by press or centrifugation method, an average volume of 2.5 L is represented by OMW (*13*). This waste matter, with a pH ranging from 4.0 to 5.3, is characterized by a dark color because of lignin components polymerized with phenolic compounds in different ways (*10*, *14*). The latter fraction, up to 10 g/L as total phenols, is especially accountable for the high toxicity of OMW together with chemical oxygen demand (COD) and biochemical oxygen demand (BOD) up to 220 g/L and 100 g/L, respectively. Also, nitrogen compounds, sugars, organic acids, and pectines are present, and the final chemical composition depends on oil extraction procedure, fruit maturation, storage time, and so forth. (*3*, *11*, *15*). Therefore, OMW toxicity is a complex property that can be related to more than one compound, even if phenols have been claimed as the most important molecules accountable for toxicity. In fact, the total phenol reduction decreases toxic potential of OMW both if it concerns the high molecular mass fraction >60 KDa (*16*) and other fractions (from 8 to 60 KDa and <8 KDa) (*9*, *15*−*18*). In an our recent study (*3*), concerning the toxicity of OMW fractioned by ultrafiltration and reverse osmosis techniques on aquatic organisms, the most toxic fraction was that from reverse osmosis (RO) containing compounds of low molecular weight (<350 Da). That investigation also provided evidence that the

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10.1021/jf0511695 CCC: $30.25
Published on Web 09/17/2005

high toxicity was prevalently due to catechol and hydroxytyrosol, the most abundant compounds of RO and constantly present in OMW. Furthermore, for the different sensitivity shown by the aquatic test organisms (algae, rotifers, and crustaceans), it was also demonstrated that a multispecies approach is required. For these reasons, toxicity studies on the main low-molecular-weight phenols, identified in RO fraction, should be enlarged to other tests that include terrestrial producers and aquatic reducers to have a complete scenario on the effects of the most toxic OMW fraction.

The aim of the present study was to acquire more comprehensive data on the ecotoxicity of the most toxic components of the OMW. For this purpose, standardized tests were used to assess the acute toxicity of the 15 compounds previously isolated from the reverse osmosis in the fractionation of OMW on the marine bacterium *Vibrio fischeri* and on the seeds of one mono- and two dicotyledons.

MATERIALS AND METHODS

Chemicals. The major phenol components of RO fraction (15 compounds) were previously isolated and were characterized as reported in Fiorentino et al. (*3*). In the present study, all products were obtained commercially. Catechol **1**, 4-hydroxybenzoic acid **2**, 3,4-dihydroxybenzoic acid (protocatechuic acid) **3**, 4-hydroxy-3-methoxybenzoic acid (vanillic acid) **4**, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid) **5**, 4-hydroxyphenylacetic acid **6**, 3,4-dihydroxyphenylacetic acid **7**, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) **8,** 4-hydroxyphenylethanol (tyrosol) **9**, 3,4-dihydroxyphenylethylene glycol **11**, 4-hydroxycinnamic acid (*p*-coumaric acid) **12,** 3,4-dihydroxycinnamic acid (caffeic acid) **13**, 4-hydroxy-3-methoxycinnamic acid (ferulic acid) **14**, and 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid) **15** were supplied by Sigma-Aldrich Chemicals while 3,4-dihydroxyphenyl ethanol (hydroxytyrosol) **10** was purchased from TCI Europe N.V., Zwijndrecht, Belgium. The structures of the compounds investigated are shown in Figure 1.

Seed Germination. The phytotoxicity of the 15 compounds was determined on two dicotyledonous species, *Cucumis sativus* and *Lepidium sativum*, and one monocotyledon, *Sorghum bicolor*. Seeds were purchased by Ingegnoli Spa (Milan, Italy). All undersized or damaged seeds were discarded, and the seeds utilized in the tests were selected for uniformity.

Bioassays were performed in compliance with the procedure of the United States Environmental Protection Agency (*19*). A range finding test was performed to determine the tolerance range of organisms to sludge-free OMW, their fractions, and pure chemicals before definitive tests were carried out to determine the 50% threshold effect.

For each experiment, 5 mL of test solution at five concentrations were added to a Petri dish (Ø 100 mm) containing a Whatman No.1 (Ø 90 mm) filter paper disk.Ten seeds were placed on each filter with four replicates for each dilution test. Controls with deionized water were conducted in parallel. The plates were incubated in a growth chamber KBW Binder 240 at 25 °C in the dark for 72 h, and then the germinated seeds (2−3 mm root and hypocotyl) were counted and the radical apparatus length was measured by means of a ruler to the closest millimeter. Furthermore, also the shoot length was measured for the *S. bicolor*. Results were evaluated in comparison with positive controls (reference toxicant $K_2Cr_2O_7$ at seven concentrations starting from 100 to 1.5625 mg CrL^{-1}).

Bacteria Test Procedure. Toxicity testing on bacteria was conducted using the Microtox test system (mod. M500 Analyzer, Azur Environmental, Carlsbad, CA) which measures the decrease in light output of the luminescent marine bacterium *V. fischeri* during a 30-min incubation period at 15 °C. Toxicants influencing the metabolism of the bacterium reduced the luminescence, which was measured at 5, 15, and 30 min and was compared to the control (*20*). Tests were carried out according to the procedure described in the Microtox Manual (*21*).

Data Analysis. Two different end points were quantified to evaluate the effect of compounds on plant physiological processes: seed germination and root elongation. A germination index (GI) was calculated by accounting for the number of grown seeds and the average sum of seed's elongation in a sample as related to the control. Results were expressed as a percentage of the control sample results according to the equation

$$GI\% = (Gs \cdot Ls)/(Gc \cdot Lc) \times 100$$

where Gs and Ls are the seed germination and root elongation (mm) for the sample, and Gc and Lc are the corresponding values for the negative controls. Furthermore, inhibition (%) values were tabulated against log-transformed data of concentrations to evaluate the test concentration corresponding to 50% germination inhibition EC_{50} with 95% confidence intervals using the software Toxcalc (*22*).

Toxic effects in light emission were expressed as median effective concentrations (EC_{50}s) for *V. fischeri* at the time when the most toxic response was obtained (30 min).

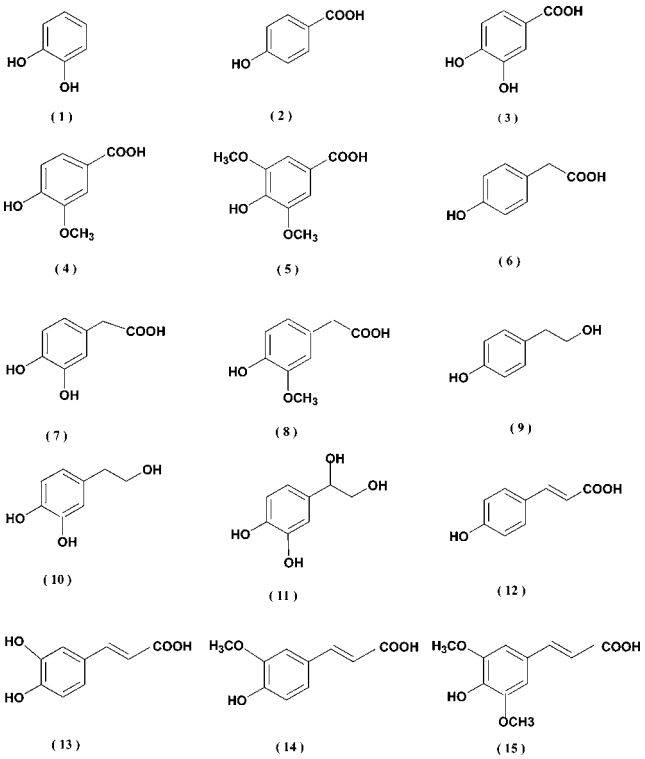

Figure 1. Structure of the compounds tested: Catechol **1**, 4-hydroxybenzoic acid **2**, 3,4-dihydroxybenzoic acid (protocatechuic acid) **3**, 4-hydroxy-3-methoxybenzoic acid (vanillic acid) **4**, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid) **5**, 4-hydroxyphenylacetic acid **6**, 3,4-dihydroxyphenylacetic acid **7**, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) **8,** 4-hydroxyphenylethanol (tyrosol) **9**, 3,4-dihydroxyphenyl ethanol (hydroxytyrosol) **10,** 3,4-dihydroxyphenylethylene glycol **11**, 4-hydroxycinnamic acid (*p*-coumaric acid) **12,** 3,4-dihydroxycinnamic acid (caffeic acid) **13**, 4-hydroxy-3-methoxycinnamic acid (ferulic acid) **14**, and 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid) **15**.

RESULTS AND DISCUSSION

The results of phytotoxicity are summarized in Table 1. For this study, the 15 selected substances were tested on three different seeds: *C. sativus* and *L. sativum* (dicotyledons) and *S. bicolor* shoot and root (monocotyledon). Phytotoxicity was tested by measuring seed germination and root elongation for all seeds chosen and measuring also the shoot growth for the *S. bicolor*. The choice of the seeds was due to previous interlaboratory exercises directed by Prof. Renato Baudo, CNR Verbania Pallanza, Italy, among more than 50 laboratories to select the most sensitive species of mono- and dicotyledons to

Table 1. Median Effective Concentration Expressed as EC_{50} (in mmol/L) for Seed Germination[a]

compounds	*Lepidium sativum*	*Cucumis sativus*	*Sorghum bicolor* root	*Sorghum bicolor* shoot
1	1.07 (0.95–1.24)	1.09 (0.82–1.49)	0.52 (0.48–0.56)	0.40 (0.38–0.45)
2	5.36 (5.35–5.37)	3.55 (3.20–3.94)	2.56 (2.40–2.72)	5.36 (5.34–5.39)
3	5.32 (4.86–5.82)	4.95 (3.85–5.53)	3.22 (2.93–3.54)	6.31 (4.99–7.98)
4	2.65 (2.58–2.72)	2.04 (1.70–2.44)	2.05 (1.83–2.34)	8.79 (7.48–10.32)
5	2.15 (2.02–2.24)	2.05 (1.92–2.10)	1.55 (1.46–1.68)	1.94 (1.86–2.02)
6	0.86 (0.79–0.96)	2.68 (2.29–3.13)	1.04 (0.95–1.15)	4.13 (3.00–5.89)
7	1.10 (0.99–1.23)	4.40 (4.02–4.81)	1.97 (1.80–2.16)	3.87 (3.27–4.57)
8	5.23 (4.90–5.58)	6.62 (5.38–8.14)	3.30 (2.93–3.72)	5.23 (4.90–5.58)
9	5.37 (5.35–5.38)	5.95 (5.04–7.02)	6.33 (4.99–8.18)	5.37 (5.34–5.39)
10	1.02 (0.88-1.15)	1.55 (1.42-1.65)	0.47 (0.45-0.52)	0.82 (0.74-0.88)
11	2.22 (2.12–2.35)	2.30 (2.17–2.38)	1.08 (0.98–1.12)	4.14 (4.08–4.25)
12	2.03 (1.81–2.27)	2.11 (1.89–2.36)	1.15 (1.03–1.29)	4.05 (3.22–5.11)
13	9.35 (7.94–11.02)	11.59 (9.67–13.90)	1.94 (1.76–2.14)	2.29 (2.07–2.34)
14	1.88 (1.83–1.93)	1.64 (1.43–1.88)	1.22 (1.16–1.29)	3.65 (2.82–4.73)
15	5.39 (5.33–5.45)	3.66 (3.34–4.00)	2.68 (2.36–3.05)	5.51 (4.64–6.54)

[a] In parenthesis 95% confidence interval.

utilize them in phytotoxicity tests (*23*). Inclusively, data revealed that the most toxic compounds were catechol **1**, with EC_{50}s ranging from 0.40 mmol/L for *S. bicolor* shoot to 1.09 for *C. sativus*, and hydroxytyrosol **10**, with EC_{50}s ranging from 0.47 mmol/L for S. *bicolor* root to 1.55 for *C. sativus* without a significant difference between the two compounds. Data for catechol agreed with other studies that showed an elevated phytotoxicity of this compound on seed germination of *Raphanus sativus*, *Triticum durum* (*24*), *Lycopersicon esculentum*, and *Cucurbita pepo* (*25*) even if these authors found higher EC_{50} values for the catechol up to 5.7 mmol/L, and 10^{-2} mol/L, respectively, probably because of the lowest sensitivity of the seeds utilized. Furthermore, present results demonstrated the major effect of catechol on the early plant growth of *S. bicolor*, suggesting that this compound may affects the OMW land disposal also when applied to newly grown seeds. These results are significant if it is considered that catechol is the most abundant compound in the most toxic fraction (RO). An analysis closely related to structure−activity among the compounds was adopted to evidence feasible effects because of functional groups. Then, the tested chemicals were grouped in five compound classes: catechol **1**, benzoic acids **2**−**5**, phenylacetic acids **6**−**8**, phenyl ethanols **9**−**11**, and cinnamic acids **12**−**15**. The present investigation, as shown in Figure 2, has revealed that the most active compounds in RO fraction were catechol, hydroxytyrosol, and cinnamic acids. The comparison of median EC_{50} values for classes differs significantly between catechol **1** and benzoic acids, catechol versus phenylacetic acids ($p < 0.05$), and catechol versus phenyl ethanols **9** and **11** ($p < 0.01$) while no statistical significance was found with cinnamic acids. A similar trend was observed for hydroxytyrosol **10** when compared with the mentioned classes ($p < 0.05$) with no difference versus cinnamic acids.

Compared to other seeds, *S. bicolor* is often especially sensitive to toxicants even if differences in sensitivity ($p < 0.05$) are revealed between *S. bicolor* root and shoot for phenylacetic acids and cinnamic acids.

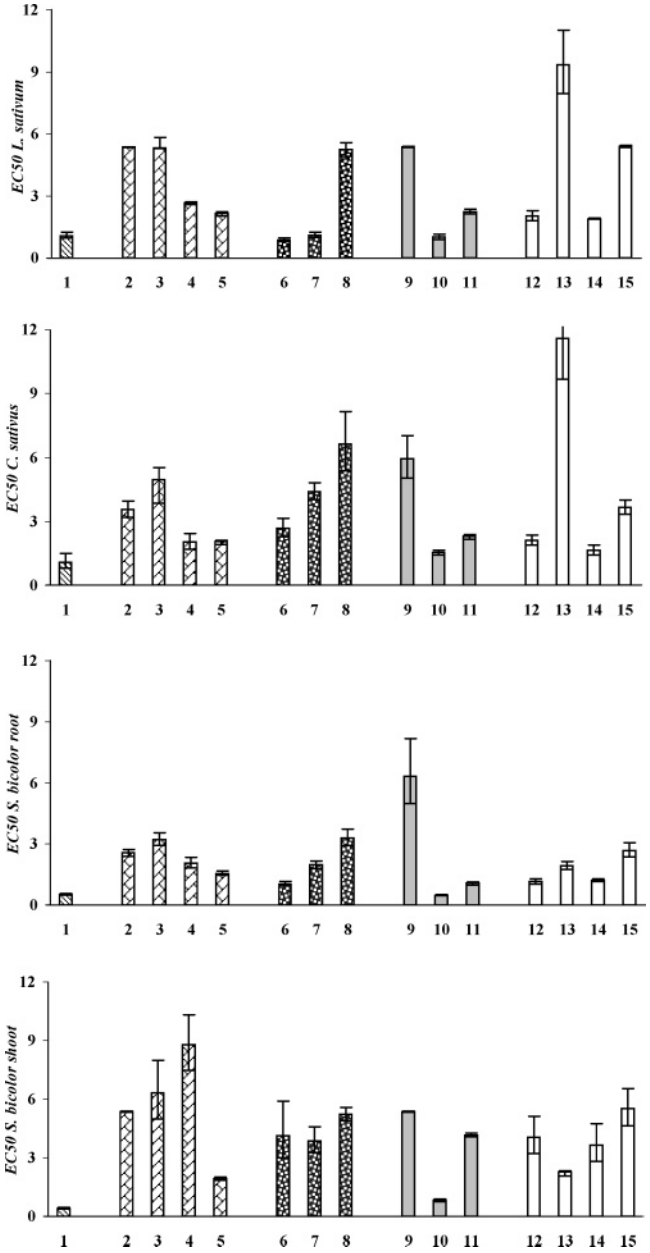

Figure 2. Phytotoxicity data of the main low-molecular-weight phenol classes, identified in RO fraction. Values of EC_{50} in mmol/L with fluctuation range for $p < 0.05$.

In this research, toxicity studies on the main low-molecular-weight phenols also included tests on the bacterium *V. fischeri*. The results are reported in Table 2 where it is evident that the bacterial toxicity is particularly elevated. In fact, EC_{50} values were 1 or 2 orders of magnitude less than phytotoxicity. The comparison of the *V. fischeri* EC_{50}s among classes differs significantly between phenylacetic acids versus phenyl ethanols and between phenyl ethanols versus cinnamic acids ($p < 0.05$). Several studies highlighted the importance to characterize the toxic potential of complex matrixes as OMW on different organisms of the food chains, comprised of the typical organisms of the freshwater system (*3*, *15*, *26*), that may be easily involved in the impact of OMW by the land runoff. Comparing the results of toxicity on the reducer *V. fischeri* and those presented in our previous study (*3*), where a multitrophic battery of aquatic organisms (producers and consumers of carbon) was used, it

Table 2. Median Effective Concentration Expressed as EC_{50} (in mmol/L) for *V. Fisheri*[a]

1	0.268 (0.232–0.310)	**6**	0.144 (0.113–0.183)	**11**	0.060 (0.048–0.085)
2	0.092 (0.065–0.131)	**7**	0.098 (0.064–0.110)	**12**	0.071 (0.041–0.125)
3	0.366 (0.334–0.400)	**8**	0.081 (0.072–0.091)	**13**	0.107 (0.092–0.143)
4	0.175 (0.141–0.217)	**9**	0.007 (0.005–0.009)	**14**	0.114 (0.092–0.140)
5	0.140 (0.118–0.155)	**10**	0.005 (0.003–0.007)	**15**	0.074 (0.061–0.089)

[a] In parentheses 95% confidence interval.

may be observed that the bacterium is the most sensitive organism to the polyphenols. In fact, for these products, acute toxicity on crustaceans and rotifers showed an EC_{50} up to 0.983 mmol/L (*3*) in opposition to the results on *V. fischeri* found in the present work where the highest observed EC_{50} was equal to 0.268 mmol/L (Table 2). These compounds are already known to possess antibacterial properties (*8*), and this result may be supported by the lower toxicity found for catechol, the only compound with one aromatic ring.

In conclusion, the experimental data have demonstrated that, when high-tech OMW treatments are not possible because of the expense and the difficulty to manage, the disposal through its spreading on land, without any pretreatment techniques, has to be carefully considered for its toxic potential on seed germination and aquatic organisms. Then, even if many countries consider irrigation of fields, with raw or pretreated OMW, an applicable system (*7*, *8*, *12*), it has always to be considered that phenolic compounds represent the most recalcitrant fraction of this effluent responsible for the major toxic properties. The results presented till now demonstrated that, although the phytotoxicity was elevated, the possible runoff of OMW would cause a stronger impact on the aquatic organisms, especially bacteria that resulted in the most susceptible to the toxic potential of this waste.

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Received for review May 20, 2005. Revised manuscript received July 15, 2005. Accepted July 17, 2005.

JF0511695